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FACTORS INFLUENCING THE IMMOBILIZATION OF GLUCOAMYLASE

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Abstract

Glucoamylase produced by *Aspergillus niger* was covalently attached to a polyacrylamide bead support possessing carboxylic functional groups activated by water-soluble carbodiimides. Factors influencing the immobilization were studied. The most favourable carbodiimide for the immobilization was N-t-butyl-N'-dimethylaminopropyl carbodiimide methyl iodide. In the experiments in which N-cyclohexyl-N'-morpholinoethyl carbodiimde methyl tosylate was used as coupling agent, the optimum medium was 0.1 M potassium phosphate buffer (pH 7.5). The support can be saturated with protein. In accordance with the molecular weight of the glucoamylase, supports with an exclusion limit of 100,000 daltons or more proved to be most advantageous.

Key words: Glucoamylase, *Aspergillus niger*, immobilization, polyacrilamide support, covalent bonds, carbodiimide effects, medium, support porosity.

Introduction

Starch is very important industrial raw material. Amylolytic enzymes play an indispensable role in its processing. α-Amylase can only be employed in soluble form, since the molecular weight of its substrates amylose and amylopectin are too high for satisfactory hydrolysis with immobilized enzymes. The second enzyme involved in the saccharification of starch is glucoamylase (1,4-α-D-glucan glucohydrolase, EC 3.2.1.3.).

Innumerable attempts have been made to immobilize this enzyme (cf. HARTMEIER, 1988). We have found that glucoamylase can be immobolized effectively by covalent attachment to a syntehic polycarboxylic matrix activated by awater-soluble carbodiimide (SZAJÁNI et al., 1985). In connection with continuous ethanol production through use of a coupled immobilized enzyme-immobilized cell reactor system, the factors influencing the immobilization of glucoamylase were studied in detail.

Materials and Methods

Materials

Glucoamylase with a specific activity of 900-1500 units/g protein was produced by *Aspergillus niger*. Akrilex C, polyacrylamide bead polymers containing carboxylic functional groups were commercial products of Reanal. Carbodiimides were synthetized according to JÁSZAY et al. (1987). Soluble starch was a preparation of E. Merck GmbH Co. (Darmstadt, Germany). All other chemicals were reagent grade commercial preparations of Reanal (Budapest, Hungary).

General methods of immobilization

Glucoamylase was covalently attached to Akrilex C bead polymers possessing carboxylic functional groups activated by a water-soluble carbodiimide, described earlier (SZAJÁNI et al., 1985). The general method of immobilization was as follows:

Akrilex C xerogel (1 g) was suspended and swollen in 50 ml of potassium phosphate buffer. The water-soluble carbodiimide, in a stoichiometric quantity relativ to the carboxylic functional groups located on the support, dissolved in 25 ml of cold (0 °C) buffer, was added with continuous stirring and cooling in an ice-bath. After 10 min, 25 ml of enzyme solution was added , and the pH was adjusted to the starting pH value. The mixture was incubated at 0-4 °C for 48 h, with two 6-h periods of agitation. The gel was filtered off by suction and successively washed three times with 100 ml of buffer, three times with 100 ml of buffer containing 1.0 M sodium chloride, three more times with 100 ml of buffer to remove unbound proteins, and finally, with a large volume of distilled water to remove the buffer ions. The products were lyophilized.

Measurement of protein

Protein determination were performed according to the method of LOWRY et al. (1951) as modified by SCHARTERLE and POLLACK (1973). The amount of immobilized protein was calculated from the difference between the amount of protein introduced into the reaction mixture and the protein present in the filtrate and washing solutions after immobilization.

Assay of glucoamylase activity

The activities of both soluble and immobilized glucoamylases were determined by measuring the amount of D-glucose liberated from soluble starch. The method routinely used was based on the iodometric titration of D-glucose (ERDEY, 1956).

In the activity test of the soluble enzyme, the reaction mixture (5.1 ml) contained 40 mg/ml soluble starch (pH 4.0) and 5-12 µg/ml enzyme. After an appropriate incubation time (30-90 min) at 60 °C, the reaction was terminated by alkali treatment. The control containing only substrate was treated in an identical manner. In the case of immobilized glucoamylase, 1.5-2.0 mg of immobilized enzyme suspended in 5.0 ml of 40 mg/ml soluble starch (pH 3.8) was stirred for an appropriate time (45-120 min) at 60 °C. The enzyme was then filtered off quickly (a few seconds) and the concentration of liberated D-glucose was determined. One unit is defined as the amount of enzyme which catalyses the liberation of one gram of D- glucose from soluble starch per hour at pH 4.0 (soluble enzyme) or pH 3.8 (immobilized enzyme) at 60 °C.

Results and discussion

Effect of carbodiimide structure on immobilization of glucoamylase

On a theoretical basis, it was supposed that disubstituted carbodiimides characterized by the general formula

$$R_1\text{-}N{=}C{=}N\text{-}(CH_2)_n\text{-}\overset{CH_3}{\underset{R_2}{\overset{|}{\underset{|}{N^+}}}}\text{-}R_3X^-$$

could effect the immobilization process. Therefore, over 30 carbodiimides bearing different substituents were synthetized and sceerned for enzyme immobilization. The catalytic activities of the immobilized enzymes were influenced advantageously by the structure of the carbodiimide used as coupling agent (SZAJÁNI et al., 1991). Data concerning the immobilization of glucoamylase are listed in Table 1. For the highest catalytic activity of the immobilized enzyme, the most favourable carbodiimide structures were those in which R_1 = tert-butyl; R_2 = methyl; R_3 = methyl ; n = 3; and X = iodide or 4-methyl-toluene sulphonate.

In the further experiments, commercially available N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate was used as coupling agent.

Effect of pH of coupling reaction mixture

In an earlier experiment (SZAJÁNI et al., 1985) in 0.1 M potassium phosphate at 0 °C, in which N-cyclohexyl-N'-morpholinoethyl carbodiimide metthyl tosylate was used, it was found that the optimum pH for the coupling is 7.5.

Effect of ionic strength of coupling reaction mixture

The effect of the ionic strength of the coupling reaction mixture was studied in potassium phosphate solution (pH 7.5) at 0-4 °C, N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate being used as coupling agent (Table 2). The ionic strength defendece shows an apparent maxmum. It is presumed that the function reflects a complex phenomenon involving changes in pH, ionozation, hydration and diffusion resistances.

Effect of protein concentration of coupling reaction mixture

The effect of the protein concentration of the coupling reaction mixture was studied in 0.1 M potassium phosphate (pH 7.5) at 0-4 °C, with N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate as coupling agent (Fig. 1). The support can be saturated with protein.

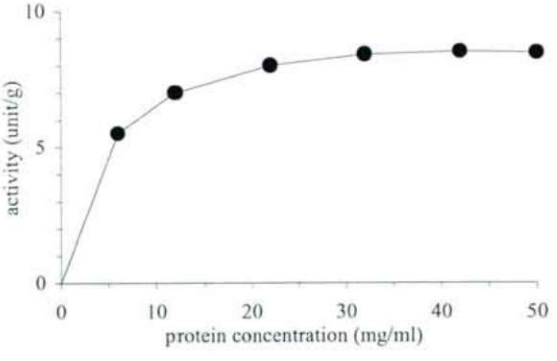

Fig. 1. Effect of protein concentration of coupling reaction mixture. Experiments were performed in 0.1 M potassium phosphate buffer (pH 7.5) at 0 °C. Akrilex C-100 xerogel (200 mg) was activated with N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate (400 mg), glucoamylase was then added.

Table 1. Effect of carbodiimide structure on immobilization of glucoamylase

Carbodiimide structure								
R_1	R_2	R_3	n	x^-	A	B	C	D
CH_3	CH_3	CH_3	2	I^-	0	0	0	0
CH_3	CH_3	CH_3	3	I^-	3.2	1.4	0.3	93.7
CH_3	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	3.8	7.2	0.4	95.6
CH_3	CH_3	C$_6$H$_5$-CH_2	3	Cl^-	0	0	0	0
CH_3	CH_3	C$_6$H$_5$-CO-CH_2	3	Br^-	1.8	96	0.2	95.8
CH_3	CH_3-CH_2	CH_3	3	I^-	0	0	0	9
CH_3-CH_2	CH_3	CH_3	2	I^-	16.3	2.6	1.3	0
CH_3-CH_2	CH_3	CH_3	3	Cl^-	13.5	13	1	21.1
CH_3-CH_2	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	15.4	2.3	1	0
CH_3-$(CH_2)_2$	CH_3	CH_3	2	I^-	0	0	0	0
CH_3-$(CH_2)_2$	CH_3	CH_3	2	H_3C-O-SO_3^-	8.6	19.1	2.6	15.7
CH_3-$(CH_2)_2$	C$_6$H$_5$-CO-CH_2	CH_3	2	Br^-	0	0	0	0
CH_3-$(CH_2)_2$	CH_3	CH_3	3	I^-	0	0	0	0
CH_3-$(CH_2)_2$	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	4.5	3	1	0
CH_3-$(CH_2)_2$	CH_3	C$_6$H$_5$-CO-CH_2	3	Br^-	0	0	0	0
CH_3-$(CH_2)_3$	CH_3	CH_3	3	I^-	17.3	82.2	3.3	1.9
CH_3-$(CH_2)_3$	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	13.7	3	1.9	9.1
CH_3-$(CH_2)_3$	CH_3	C$_6$H$_5$-CO-CH_2	3	Br^-	10	11.5	3	20.1
H_3C-C(CH_3)(CH_3)-	CH_3	CH_3	3	I^-	41.6	10	3.8	5.7
H_3C-C(CH_3)(CH_3)-	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	37.8	5	4.2	2.2
C$_6$H$_5$-	CH_3	CH_3	2	I^-	17.8	7.4	3.8	26.3
C$_6$H$_5$-	CH_3	CH_3	2	H_3C-C$_6$H$_4$-SO_3^-	8.9	13.5	2.7	30.3
C$_6$H$_5$-	CH_3	CH_3	3	I^-	11	12	5	11.8
C$_6$H$_5$-	CH_3	CH_3	3	H_3C-C$_6$H$_4$-SO_3^-	9	-	2.8	0
C$_6$H$_5$-	CH_3	C$_6$H$_5$-CO-CH_2	3	Br^-	15.8	2.6	1.5	31.8
C$_6$H$_5$-	CH_3	O_2N-C$_6$H$_4$-CO-CH_2	3	Br^-	1.8	-	0.2	95.8
C$_6$H$_5$-	CH_3-N^+=C$_5$H$_5$ O^-		2	I^-	16.3	6.1	1.7	32.7
C$_6$H$_5$-	CH_3-N^+=C$_5$H$_5$ O^-		2	H_3C-C$_6$H$_4$-SO_3^-	17.7	5	2.2	7.8
C$_6$H$_5$-	CH_3-N^+=C$_5$H$_5$ O^-		3	I^-	22.3	8.3	2.5	9.9
C$_6$H$_5$-	CH_3-N^+=C$_5$H$_5$ CH_3		3	I^-	17.3	4	1.2	0
C$_6$H$_5$-	CH_3-N^+=C$_5$H$_5$ CH_3		3	H_3C-C$_6$H$_4$-SO_3^-	17.3	8.3	2.3	0
C$_6$H$_5$-CH_2-	CH_3	CH_3	3	I^-	0.5	0.5	0.1	98
C$_6$H$_5$-	CH_2-CH_3 N^+-CH_2-CO-C$_6$H$_5$ CH_2-CH_3		3	Br^-	17.3	18.7	1.4	47.2

A: Activity on dry wt basis (units/g solid); B: Activity on protein basis (%)[a]; C: Activity bound (%)[b]; D: Activity loss (%)[b].

[a] The activity of the soluble enzyme was taken as 100%.

[b] The total activity introduced into the coupling reaction mixture was taken as 100%.

Effect of porosity support on immobilization of glucoamylase

In a study of the effect of the support potosity on the immobilization of glucoamylase, a series of Akrilex C bead polymers characterized by the molecular exclusion limit were used.

Experiments were performed in 0.1 M potassium phosphate buffer (pH 7.5) at 0-4 ^{o}C, with N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate as coupling agent (Table 3). The results were in good agreement with the data concerning the molecular weigth of *Aspergillus niger* glucoamylase (FOGARTY and BENSON, 1983).

Table 2. Effect of ionic strength of coupling reaction mixture on immobilization of glucoamylase[a].

Ionic strength	Activity (units g^{-1} solid)
0.127	1.3
0.052	1.9
0.134	5.8
0.268	10.9
0.536	4.2

[a]Experiments were performed in potassium phosphate buffer (pH 7.5) at 0-4 °C. Akrilex C-100 xerogel (200 mg) was activated with N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate (400 mg), and glucoamylase (250 mg) was then added.

Table 3. Effect of porisity of support on immobilization of glucoamylase[a].

Support	Exclusion limit (dalton)	Activity (units g^{-1} solid)
Akrilex C-30	30,000	5.1
C-60	60,000	5.8
C-100	100,000	9.5
C-200	200,000	9,7

[a]Experiments were performed in 0.1 M potassium phosphate buffer (pH 7.5) at 0-4 ^{o}C. Akrilex C xerogel (200 mg) was activated with N-cyclohexyl-N'-morpholinoethyl carbodiimide methyl tosylate (400 mg), and glucoamylase (250 mg) was then added.

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